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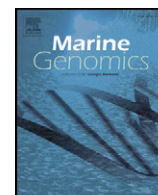

Molecular characterization and mRNA expression of catalase from pearl oyster *Pinctada fucata*

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ABSTRACT

Catalase (EC 1.11.1.6) is an important antioxidant enzyme that protects aerobic organisms against oxidative damage by degrading hydrogen peroxide to water and oxygen. In the present study, a catalase cDNA of peal oyster *Pincatada fucata* (designated as PoCAT) is cloned and characterized by expressed sequence tag (EST) and rapid amplification of cDNA ends (RACE) methods. PoCAT is 2428 bp long and consists of a 5′-UTR of 140 bp, an unusually long 3′-UTR of 749 bp, and an open reading frame (ORF) of 1539 bp. The ORF of PoCAT encodes a polypeptide of 512 amino acids with molecular weight of 58.1 kDa and the theoretical isoelectric point of 8.4. PoCAT shares 62.3–82.2% identity and 73.0–92.0% similarity to other catalase amino acid sequences. Sequence alignment indicates that PoCAT contains the proximal heme-ligand signature sequence ($R^{351}LFSYSDT^{358}$), the proximal active site signature ($F^{61}NRERIPERVVHAKGGGA^{78}$), and the three catalytic amino acid residues (His^{72}, Asn^{145}, and Tyr ^{355}). PoCAT has two potential glycosylation sites ($N^{436}YS^{438}$ and $N^{478}FS^{480}$) and a peroxisome targeting signal (ASL). PoCAT mRNA was ubiquitously expressed in all detected tissues, and the expression level of PoCAT mRNA was higher in intestine and mantle. The expression profile analysis showed that the expression level of PoCAT mRNA in intestine was significantly up-regulated at 2, 4 and 12 h after *Vibrio alginolyticus* stimulation. These results demonstrated that PoCAT is a typical member of catalase family and might be involved in innate immune responses of pearl oyster.

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1. Introduction

Reactive oxygen species (ROS) such as superoxide anion ($O_2{\bullet}^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH{\bullet}^-$) naturally occur in organisms that use molecular oxygen for production of energy (Li et al., 2008). ROS can stimulate signal transduction pathways like NF-κB or MAPK (Morey et al., 2001; Nakano et al., 2006; Pantano et al., 2006) and mediate cell growth and apoptosis (Suzuki et al., 1997; Lesser, 2006). ROS also plays an important immune defense against infection during phagocytosis. When the organism is attacked by microorganism, phagocytosis is activated in the host with high oxygen consumption followed by numerous ROS production, which can kill foreign invaders (Schwarz, 1996; Bogdan et al., 2000; Xiang, 2001). However, the mass accumulation of ROS has detrimental effects on surrounding cells, and oxidative stress caused by the excessive ROS can lead to lipid peroxidation, protein oxidation, DNA damage, membrane disruption, and mitochondrial dysfunction (Li et al., 2008; Griswold et al., 1993; Bestwick and Maffulli, 2004; Yamamoto et al., 2005) Consequently, to maintain ROS at suitable levels is essential for normal cell function and organism survival. Organisms develop complex antioxidant defense systems for protection against oxidative stress, such as glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), and non-enzymatic components (β-carotene, glutathione, and α-tocopherol) (Mittapalli et al., 2007).

Catalase is an important antioxidant enzyme and exists virtually in all oxygen-respiring organisms as a hemoprotein with four identical subunits of approximately 50–60 kDa (Zhang et al., 2008). It mediates antioxidant defense reactions by catalyzing the reduction of toxic hydrogen peroxide (H_2O_2) into oxygen and water (Zhang et al., 2008; Nordberg and Arner, 2001). Recently, an extracellular immune-regulated catalase of Drosophila was demonstrated to mediate a key host defense system, which was needed during host–microbe interaction in the gastrointestinal tract (Ha et al., 2005; Ryu et al., 2006). So far, a number of catalase genes have been cloned and characterized from bacteria, plant and animal (Li et al., 2008; Yamamoto et al., 2005; Glenn et al., 2000). However, in mollusks, sequence information of catalase gene was only limited to a few mollusk species, such as *Chlamys farreri* (DQ862859), *Haliotis discus discus* (DQ530211), *Crepidula fornicata* (DQ087480), *Modiolus americanus* (AY580307), and *Nucula proxima*

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(AY580231). To further know the functions and molecular evolution of catalase in mollusk, it is necessary to clone and characterized more catalase cDNA from various species.

Pearl oyster *Pinctada fucata* is an important bivalve mollusk for seawater pearl production in China. However, with the rapid development of industry and frequent anthropogenic pollution in the last decades, outbreaks of disease and ocean pollution have affected the pearl aquaculture industry, and lead to heavy economic losses. Pearl oyster has suffered serious disease caused mainly by bacteria (Lau et al., 2006), rickettsia-like organism (Wu and Pan, 1997), parasites (Hine and Thorne, 2000; Spiers et al., 2008) and virus (Suzuki et al., 1998; Kitamura et al., 2000, 2002), which could be related to the dramatic decline in South China seawater pearl production. In recent years, to control disease and enhance the yields and quality of seawater pearl, great effort has been done to understand the molecular mechanism of immune regulation of pearl oyster, and some immune-relevant factors have been identified and characterized from pearl oyster (Zhang et al., 2009a,b,c, 2010). In the present study, we cloned the full-length cDNA of catalase from pearl oyster *P. fucata* (designated as PoCAT) and investigated the expression pattern of its mRNA in different tissues and temporal expression profile after bacterial challenge.

2. Materials and methods

2.1. Pearl oyster and immune challenge

Pearl oyster *P. fucata* (body weight 18.2–22.5 g) was obtained from pearl oyster culture base of South China Sea Fisheries Research Institute in Xincun village, Hainan province, China and maintained at 25–27 °C in tanks with recirculating seawater for one week before experiment. The pearl oyster was fed twice daily on *Tetraselmis suecica* and *Isochrysis galbana* in the whole experiment process. Pearl oysters were injected into the adductor muscle with 100 μl of Phosphate Buffered Saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) as control group. The bacterial challenge group was performed by injecting with 100 μl of *Vibro alginolyticus* resuspended in PBS to $OD_{600} = 0.4$ (1 OD $= 5 \times 10^8$ bacteria ml^{-1}) into the adductor muscles of each pearl oyster. At each time point (0, 2, 4, 8 and 12), intestine was collected from control group and bacterial challenge group and stored in liquid nitrogen until used. For tissue distribution analysis, unchallenged pearl oyster's digestive gland, gills, mantle and intestine, and gonad were collected as unchallenged group and stored in liquid nitrogen until used. Pearl oysters of each group were divided into three replicates with equal amounts and fed in three tanks. Five pearl oysters were randomly sampled from each group at each time point, and mixed corresponding tissues with equal amounts as one sample.

2.2. cDNA library construction and EST analysis

A cDNA library was constructed from the whole body of a pearl oyster challenged by *V. alginolyticus*, using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII Gold cloning kit (Stratagene). Random sequencing of the library using T3:AATTAACCCTCACTAAAGGG yielded 6741 successful sequencing reactions, which were clustered into 808 contigs and 2456 singlets. BLAST analysis of all expressed sequence tag (EST) sequences revealed that an EST of 583 bp (EST no: Pmpca0_000207) was homologous to the catalase of *C. farreri* (ABI64115) and *Cristaria plicata* (ADM64337.1). Based on the sequence of this EST, the corresponding colony was picked up and resequenced to obtain the complete sequence of the catalase.

2.3. Sequence analysis of PoCAT

PoCAT amino acid sequence was predicted using DNATool version 6.0 software. The percentage of similarity and identity of the known catalase sequences was calculated using the MatGAT program (Campanella et al., 2003) with default parameters. The protein domain was predicted with the simple modular architecture research tool (SMART) program (Schultz et al., 1998; Letunic et al., 2006). The protein sequence of catalase was compared to its counterpart sequences currently available in GenBank using BLAST program (Altschul et al., 1997) (http://www.ncbi.nlm.nih.gov). Multiple alignment of catalase was carried out with ClustalW program (http://www.ebi.ac.uk/clustalw/). The phylogenetic tree was constructed with MEGA program version 3.1 (Tamura et al., 2007) based on amino acid sequence alignment. The phylogenetic tree was tested for reliability using 1000 bootstrap replications. The secondary structure is predicted using Predictprotein (http://www.predictprotein.org) software. To generate the PoCAT 3D structural model, the deduced amino acid sequence was submitted to Swiss-Model (http://swissmodel.expasy.org//SWISS-MODEL.html). The prediction was conducted in first approach mode with default parameters (Schwede et al., 2003).

2.4. Quantitative RT-PCR analysis of PoCAT

The expression pattern of catalase in digestive gland, gonad, gills, mantle and intestine from unchallenged group was detected by quantitative RT-PCR. Temporal expression level in intestine after bacterial challenge was also detected by quantitative RT-PCR. Total RNA samples were extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions, and treated with DNase I (QIAGEN) to remove contaminated DNA. Subsequently, the first-strand cDNA was synthesized based on manufacture's instruction of PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa) using total RNA as template. cDNA mix was diluted to 1:5 and stored at −80 °C for subsequent Quantitative RT-PCR. Two catalase gene-specific primers, PoCAT-F (5′-AAGAAGGAGGCT-GATTGA-3′) and PoCAT-R (5′-CACCTACCATTTCCCACA-3′), were designed to amplify a product of 264 bp. The β-actin gene was used as an internal control to verify the real-time quantitative RT-PCR reaction and adjust the cDNA templates. Two β-actin gene-specific primers, β-actin-F (5′-GCCGAAAGAGAAATCGTCAG-3′) and β-actin-R (5′-TGGCTGGAATAGGGATTCTG-3′), were designed to amplify a fragment of 183 bp.

Quantitative RT-PCR was performed in a total volume of 20 μl containing 10 μl of 2 × SYBR Green Real-time PCR Master Mix (TaKaRa DRR041A), 1 μl of cDNA, 0.16 μM of each primer and 8.2 μl of double-distilled water. Quantitative RT-PCR program consisted of denaturation step at 96 °C for 2 min, followed by 40 amplification cycles of 15 s denaturation at 96 °C, 15 s annealing at 54.6 °C, and 30 s extension at 72 °C. Fluorescence readings were performed at the end of each cycle. To analyze the PoCAT mRNA expression level, the comparative CT method ($2^{-\Delta\Delta CT}$ method) was used. The CT for the target amplified PoCAT and the CT for the internal control β-actin were determined for each sample. Differences in the CT for the target and the internal control, called ΔCT, were calculated to normalize the differences in the amount of total cDNA added to each reaction and the efficiency of the RT-PCR. The control group was used as the reference sample, called the calibrator. The ΔCT for each sample was subtracted from the ΔCT of the calibrator, the difference was called ΔΔCT. The PoCAT mRNA expression level could be calculated by $2^{-\Delta\Delta CT}$, and the value stood for an n-fold difference relative to the calibrator.

2.5. Statistical analysis

Statistical analysis was carried out with GraphPad Prism 5.0 software. The normality of the distribution and homogeneity of variances of data were examined by Shapiro–Wilks and Cochran tests, and then data were analyzed by one-way analysis of variance (ANOVA) with default parameters to identify differences between groups. Differences were considered statistically significant when p values were lower than 0.05.

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